Journal of Toxicology and Environmental Health Sciences Vol. 3(3) pp. 80-94, March 2011
Available online at http://www.academicjournals.org/JTEHS
ISSN 2006-9820 ©2011 Academic Journals

Full Length Research Paper

Toxicologic characterization of a novel explosive, triaminoguanidinium-1-methyl-5-nitriminotetrazolate (TAG-MNT), in female rats and *in vitro* assays

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Accepted 10 January, 2011

Sustainable use of training ranges requires the development of compounds that have a minimal impact on the environment when used in a weapon system. Triaminoguanidinium-1-methyl-5-nitriminotetrazolate (TAG-MNT) is a novel, explosive, military compound of interest for application in some weapon systems. Little is known of its toxicologic properties. To ensure the health of potentially exposed personnel and the environment, several initial toxicity investigations were conducted and the results compared with another widely used energetic (hexahydro-1,3,5-trinitro-1,3,5-triazine; RDX). In a novel microplate Ames assay, TAG-MNT was a weak mutagen only at the limit concentration of 2 g/L. However, TAG-MNT was cytotoxic to bacteria and a human liver cell line at 250 mg/L and greater. Unlike RDX, TAG-MNT did not have an affinity for the GABAa receptor convulsant site, and was predicted not to induce seizure. After acute oral dosing in female rats, TAG-MNT had no apparent adverse effect up to the limit dose of 2 g/kg. However, daily oral dosing for 14 days at exposures of 1000 mg/kg-d and above caused reduction in food intake, weight loss, increased kidney weight, leucopenia, and elevated blood urea nitrogen and creatinine levels. Leucopenia, increased liver mass, evidence of liver hepatocyte necrosis and centrilobular hypertrophy were observed at 500 mg/kg-d and above. TAG-MNT was negative in the rat micronucleus assay of blood samples. Based on these data, the 14-day oral No observed adverse effect level (NOAEL) and the lowest observed adverse effect level (LOAEL) is 250 and 500 mg kg^{-l} day^{-1}, respectively.

Key words: RDX, Ames assay, cytotoxicity, oral toxicity, rat micronucleus assay

INTRODUCTION

Current land use patterns and expanding suburban populations present encroachment issues for many military installations. Although there have been efforts to close and realign many installations, wise stewardship of remaining active training sites is a priority of the Army. Therefore, the development of important environmental and occupational toxicity data are important for new compounds; early in development, decisions can be made regarding further testing and implementation (American Society for Testing and Materials (ASTM), 2008).

One novel compound, triaminoguanidinium-1-methyl-5-nitriminotetrazolate (TAG-MNT) is being developed for use as an energetic (Hammer et al., 2005; Klapötke et al., 2008; Klapötke et al., 2008). There is essentially no information on the toxicologic properties of nitriminotetrazolates and TAG-MNT in particular. Several studies were initiated to evaluate the toxicity of this compound. These studies included: determination of

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Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
JAN 2011		**00-00-2011 to 00-00-2011**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Toxicologic characterization of a novel explosive, triaminoguanidinium-1-methyl-5-nitriminotetrazolate (TAG-MNT), in female rats and in vitro assays	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **US Army Public Health Command (Provisional) [USAPHC (Prov)],Directorate of Toxicology,Aberdeen Proving Ground,MD,21010**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT **unclassified**	b. ABSTRACT **unclassified**	c. THIS PAGE **unclassified**	**Same as Report (SAR)**	**15**	

TAG-MNT mutagenicity using a microplate Ames assay; evaluation of its ***in vitro*** cytotoxicity in bacteria by measuring luminescence (e.g., ATP assay and Microtox® test) and in a human liver cell line by neutral red uptake (NRU) assay; and evaluation of its ***in vivo*** acute and sub-acute oral toxicity in female, Sprague-Dawley rats. An ***in vivo*** genotoxicty assay was also included as part of the oral 14-days exposures (that is, rat micronucleus assay).

MATERIALS AND METHODS

Test compound

The TAG-MNT was synthesized and obtained from the Army Research Development and Engineering Center (ARDEC, Picatinny Arsenal, NJ). The lot number was identified as RDD09A004E001 with a purity of 98 to 99% by weight as measured by NMR spectral analysis (R. Damavarapu, personal communication). The Chromatographic Analysis Division (Explosives Team), Directorate of Laboratory Sciences (DLS; USAPHC (Prov), Aberdeen, MD) developed an analytical method for TAG-MNT using high performance liquid chromatography with ultraviolet detection at 313 nm. The system employed separation on a reverse phase C-18 column (150 × 4.6 mm) with a mobile phase of 75/25 acetonitrile and water at a flow rate of 1.0 ml/min. The concentration ranged from 1 to 100 µg/ml in an injection volume of 100 µl. TAG-MNT had a retention time of 1.52 min.

TAG-MNT is readily soluble in water up to 98.3 mg/ml at 22°C (R. Pesce-Rodriguez, personal communication). For the ***in vitro*** microplate Ames assay, a 25-X stock solution of TAG-MNT was prepared (50 mg/ml in sterile water). For the ***in vivo*** oral toxicity studies, intended doses of 2000 mg/kg required the use of supersaturated suspensions of TAG-MNT as the maximum daily oral dose volume could not exceed 10 ml/kg. For the ***in vivo*** sub-acute experiments, 1% methylcellulose, 0.2% Tween 80, in tap water was used as the vehicle; the methylcellulose (CAS # 9004-67-5, lot number 037690), and the Tween 80 (CAS # 9005-65-6 lot number 032097) were purchased from Fisher Scientific (Fairlawn, NJ). Suspensions and solutions of TAG-MNT were verified to be homogenous and stable at 22°C up to 3 weeks.

GABAa receptor binding assay

TAG-MNT was tested at a single concentration of 33 µM for affinity to the GABAa convulsant site. The [35S]-TBPS convulsant site assay, based on the method of Maksay (1993) was performed by MDS Pharma Services, (King of Prussia, PA) using picrotoxin as a standard.

In vitro microplate Ames assay

A microplate Ames assay (Xenometrics MPF™ Ames Assay, AG, Switzerland) was used that provided a convenient, high throughput capability for mutagenicity testing (Xenometrix MPF™ is a trademark of Xenometrix AG, Switzerland). The test uses the ***Salmonella typhimurium*** strains TA98, TA100, TA1535 and TA1537, and ***Escherichia coli*** strains WP2 uvrA and WP2 [pKM101] of bacteria (Kamber et al., 2009; Umbuzeiro et al., 2010).

Briefly, bacterial suspensions were diluted with incubation medium and aliquoted to tissue culture wells containing serial dilutions of the 50 mg/ml, 25-X concentrate of TAG-MNT. The bacterial suspensions were incubated with shaking for 90 min at 37°C with and without the inclusion of Aroclor-induced, S9 liver extract (±S9). At the end of the incubation, each well was diluted 11-fold with purple indicator medium and 50 µl aliquots were distributed appropriately into 384-well plates. These plates were incubated anaerobically for 2 days at 37°C and then scored for presence of revertant colonies, that is, evidence of a positive mutagenic event. The colormetric indicator reacts to changes in pH resulting from metabolism by living, mutated bacteria. Positive wells change from purple to yellow and are scored as a mutagenic event.

A positive control appropriate for each strain was run coincidently with TAG-MNT to assure the assay was valid for each trial: 2 µg/ml 2-nitrofluorene TA98 –S9; 0.1 µg/ml 4-nitroquinoline-N-oxide TA100 –S9; . 100 µg/ml N4-aminocytidine TA1535 –S9; 15 µg/ml 9-aminoacridine TA1537 –S9; 1 µg/ml 4-nitroquinoline-N-oxide ***E. coli*** -S9; 5 µg/ml 2-aminoanthracene TA98, TA100, TA1535 and TA1537 +S9; and 50 µg/ml 2-aminoanthracene ***E. coli*** +S9. The assay as a whole is determined to be valid if the number of control background reversions and the number of positive control reversions is within prescribed limits (Xenometrix 2009). A positive result was indicated if the number of reversions induced by the test compound was at least 2-fold above the background control, and there is a concentration-related increase over the range tested and/or a reproducible increase at one or more concentrations in the number of revertant colonies per plate in at least one strain with or without metabolic activation system (Xenometrix, 2009).

In vitro cytotoxicity assays

ATP assay

Coincident with the mutagenic incubation, a duplicate plate was prepared for determination of cytotoxicity of TAG-MNT using ATP luminescence. After the 90 min incubation at 37°C, samples from the incubation plate were aliquoted to a 96-well plate. An equal volume of luminescent reagent was added to each well according the method described for the BacTiter-Glo™ microbial cell viability assay (BacTiter-Glo™ is a trademark of Promega Corporation Madison, WI). Luminescence was measured using a synergy TM HT multi-detection microplate reader (Model SIAFRTD) and Gen5TM software (BioTek Instruments Inc., Winooski, VT). Cytotoxicity of TAG-MNT in the BacTiter-Glo assay was indicated when the luminosity of ATP in compound-treated cultures was decreased below the levels in vehicle-treated cultures; the level of ATP generated luminosity correlates with the number of living bacteria. Data are expressed as a percentage of the level of luminosity generated by the control, vehicle-treated bacteria.

Microtox® acute toxicity test

Cytotoxicity of TAG-MNT to the marine bacterium, ***Vibrio fischeri*** was evaluated using a Microtox Model 500 Analyzer (SDIX Strategic Diagnostic, Inc.; (Choi and Meier, 2001). ***V. fischeri*** NRRL-B-11177, BSL-1 (AZF686018A) was purchased from SDIX. Loss of luminescence, indicating a loss or decrease in cell viability and cell death, was recorded at 5, 15 and 30 min; EC50 values at 5, 15, and 30 min were determined by the MicrotoxOmni™ software.

Neutral red uptake assay

The neutral red uptake assay was run in accordance with National Institute of Environmental Health Sciences guidelines (Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) et al., 2006). Cells of human liver origin (Chang liver CCL-13, ATCC), were seeded into 96-well plates at 5.0×10^3 cells/well/0.1 ml and maintained in culture for 24 h to form a semi-

confluent monolayer. The cells were treated with TAG-MNT over a range of 8 concentrations for 48 ± 1 h in 37°C, 5% CO_2 incubator. Treatment medium was removed and the cultures were washed once with phosphate buffered saline (PBS). Neutral red medium (NRM containing 33 µg dye/ml) was added to each well (0.2 ml/ml/well). After 3 h incubation, NRM was discarded and the cultures were washed once with PBS and received 0.1 ml of NR desorbing fixative per well. The plates were placed on a shaker for 20 min at room temperature (24 ± 2°C). NR absorption was detected at optical density (OD) 540 nm using a synergy™ HT multi-detection microplate reader (Model SIAFRTD) and Gen5™ software (BioTek Instruments Inc., Winooski, VT).

Animals

Oral toxicity studies were conducted using young adult female Sprague-Dawley rats obtained from Charles River Laboratories (Wilmington, MA). Rats were 8 weeks old and six weeks old for the acute and 14-day subacute toxicity studies, respectively. The attending veterinarian examined the animals and found them to be in acceptable health. The animals were quarantined for a minimum of 5 days after arrival. All rats were maintained in a temperature-, relative humidity- and light-controlled room. The conditions were 64 to 79°F, 30 to 70% relative humidity with a 12-h light/dark cycle. A certified pesticide-free rodent chow (Harlan Teklad®, 8728C Certified Rodent Diet) and drinking quality water were available ***ad libitum*** (® Teklad Certified Rat Diet is a registered trademark of Harlan, Teklad, Madison, Wisconsin). Rats were housed individually in suspended polycarbonate boxes with Harlan Sani-Chip® bedding (® Harlan Sani-Chip is a registered trademark with P. J. Murphy Forest Products Corporation, Montville, New Jersey). Each rat was uniquely identified by number using cage cards only for the acute study and both cage cards and microchip implants (BioMedic Data Systems, Inc., Seaford, DE) for the 14-day study.

Acute study (Sequential Stage-Wise Probit, SSWP)

The objective of this study was to determine the acute oral LD50 of TAG-MNT in the female Sprague-Dawley rat using the SSWP (Feder et al., 1991; Feder et al., 1991), and to guide oral exposures for the subacute (14-day) study. The general procedures of this acute study followed the USEPA Health Effects Test Guidelines for Acute Oral Toxicity (OPPTS 870.1100) (USEPA, 1998). Tests were performed using two separate stages of dosing.

All animals were fasted overnight prior to dosing and for up to 4 h post-dosing. Doses for the first stage of the acute tests were 180, 270, 400, 600, 900, 1350 and 2000 mg/kg. All doses were calculated based on body weights taken immediately prior to dosing. The amount of TAG-MNT appropriate for each rat was weighed individually in a weigh pan, suspended in corn oil and administered by oral gavage using a 16 gauge × 2-inch stainless steel gavage needle; maximum volume did not exceed 10 ml/kg. In the second stage, two groups of 3 female rats received 2000 mg TAG-MNT/kg body mass. During the course of the acute study, it was determined that the preferred vehicle for the 14-day, sub-acute study would be 1% methylcellulose, 0.2% Tween 80, in tap water; suspension of TAG-MNT was prolonged and the TAG-MNT more soluble in the methylcellulose/Tween 80/ tap water vehicle. To rule out an effect of vehicle on possible toxicity of TAG-MNT, the corn oil vehicle was repeated coincidently with a second group dosed with TAG-MNT suspended in methylcellulose.

Following the administration of the test compound for each stage of the acute test, the rats were observed for 14 days. All clinical signs or incidences of death were recorded on a daily basis. Individual body weights were recorded daily (5 days a week) throughout the 14-day observation period to determine recovery. Surviving animals were euthanized on day 14 and necropsied for gross pathological examination.

14-Day oral repeated dose toxicity study

Seventy female Sprague-Dawley rats were randomly distributed into seven treatment groups consisting of 10 rats each. The animals were then divided into three evenly distributed experimental groups; the start dates for each group were staggered over a period of three days to facilitate scheduling of necropsies. On the morning of each day, each rat received either 0 (methyl cellulose and water vehicle control), 62.5, 125, 250, 500, 1000, or 2000 mg TAG-MNT/kg body mass-day via gavage using a 16 gauge × 2-inch stainless steel gavage needle. Similar volumes of dosing solutions were administered to all animals using three dosing solutions at concentrations of 2000, 500 and 125 mg/L which resulted in dosing volumes of either 10 or 5 ml/kg. The control animals received the same volume per body weight as the highest dosage group, that is, 10 ml/kg. The doses were administered daily, 7 days per week (total of 14 doses) for the 14-day study. The solution/suspensions were sampled and analyzed to verify concentrations and stability prior to the first day of exposure.

Body weights and feeder weights were recorded on days 0, 1, 3, 7 and 14. Animals were observed daily for toxic signs and morbidity. Water consumption was not monitored during this study.

Following the 14-day study period, the rats were anesthetized with isoflurane gas. Blood was collected by intracardiac puncture and the rats were euthanized using carbon dioxide. Clinical chemistry and hematology values were determined from all valid samples. The adrenals, brain, heart, kidneys, liver, ovaries, spleen, thymus, and uterus were removed and weighed for absolute organ weights, organ-to-body weight ratios, and organ-to-brain weight ratios. Gross necropsies were completed on all terminal animals. The following parameters, by test group, were analyzed and compared to the controls: Body weights; weight gains; food consumption; absolute organ weights; organ-to-body weight ratios; and organ-to-brain weight ratios.

Hematology on blood samples was accomplished using a Cell-Dyn 3700 Hematology Analyzer (Abbott Laboratories, Abbott Park, Illinois). Parameters measured included: White blood cell count (WBC), WBC differential (% neutrophils (NEU, %N), % lymphocytes (LYM, %L), % monocytes (MONO, %M), % eosinophils (EOS, %E), % basophils (BASO, %B)), red blood cell count (RBC), hemoglobin (HGB), hematocrit (HCT), mean cell volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC), red blood cell distribution width (RDW), platelets (PLT), and mean platelet volume (MPV).

Clinical chemistry was accomplished using a VetTest 8008 chemistry analyzer and VetLyte Na, K, Cl analyzer (IDEXX Laboratories, Inc., One IDEXX Drive, Westbrook, ME). Parameters measured included albumin (ALB), alkaline phosphatase (ALK P), alanine aminotransferase (ALT), blood urea nitrogen (BUN), calcium (Ca), cholesterol (CHOL), creatinine (CREA), glucose (non-fasting) (GLU), globulin (GLOB), lactate dehydrogenase (LDH), phosphorus (PHOS), total bilirubin (TBIL), total protein (TP), sodium (Na), potassium (K), and chlorine (Cl).

Genotoxicity to rat peripheral blood was evaluated using a micronucleus assay. Following the 14-day study period, blood was collected from the lateral saphenous vein of animals from the top three surviving dose groups (250, 500, 1000 $mgkg^{-1}day^{-1}$), vehicle control, untreated control, and positive control groups. A positive control group (n = 10) was given three oral doses (48, 24, and 4 h prior to sacrifice) of ethylmethane sulfonate at 200 mg/kg. The micronucleus assay was conducted on peripheral blood using the MicroFlow Plus Kit® (Litron Laboratories) following the manufacturer's instructions. Briefly, blood was placed in anticoagulant, fixed in ultracold methanol, and store at -75 to -85°C

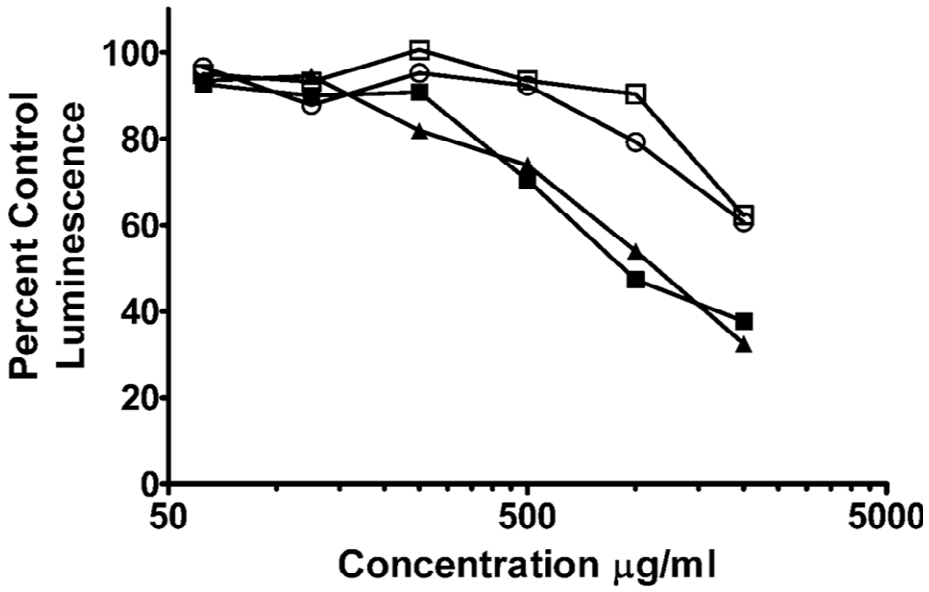

Figure 1. Effect of TAG-MNT on viability of bacteria. ATP luminescence was measured after 90 min incubation at 37°C with increasing concentrations of TAG-MNT. Decreased luminescence from the level expressed in control cultures is evidence of cytotoxicity. The LD_{50} is estimated to range from 1 to 2 g/L depending on bacterial strain. ■ – TA98, ▲ – TA 100, ○ – TA1535, □ – ***E. coli.*** Cytotoxicity in TA1537 strain was not evaluated.

until analysis. On the day of analysis by flow cytometry, the blood samples were washed with PBS to remove fixative, treated with RNase A, labeled with anti-CD71-FITC and anti-CD61-PE, and then stained with DNA staining solution (propidium iodide, PI). Prior to analysis of samples, the flow cytometer (Beckman Coulter EPICS XL) was calibrated using the kit provided standards. Anti-CD71, anti-platelet–PE and PI fluorescence signals were detected in the FL1, FL2 and FL3 channels, respectively. Micronucleated reticulocytes (MN-RET) were identified as those that show both CD71 and PI-associated fluorescence. A total of 20,000 MN-RETs were analyzed per sample. The data collected from the micro-nucleus assay were expressed as the percentage of reticulocytes with micronuclei (%MN-RET) and the percentage of red blood cells that were reticulocytes (%RET).

Statistical analyses

Data were analyzed using a one-way ANOVA. If significant, a post hoc Dunnett's multiple comparison test was used to compare dose groups to the control group. Statistical significance was defined at the $p < 0.05$ level. To allow for a consistent characterization of the results, means and standard deviations are presented for all data. Tests were conducted using Prism 4 (GraphPad Software, La Jolla, CA).

For the micronucleus assay, a one-way analysis of variance (ANOVA) was used to test for significant differences in %MN-RET and %RET. The Tukey multiple comparison test was used to evaluate the differences between groups. The results were considered to be statistically significant at $p< 0.05$. SPSS® version 16.0 (SPSS Inc., Chicago, IL) was used for all analyses.

Histopathology

Selected samples of liver and kidney from each dose group (n = 5 per group) were collected, trimmed, fixed in formalin, and embedded in paraffin. These tissues were then sectioned at 6 microns, stained with hematoxylin and eosin, and examined via light microscopy.

This study was conducted consistent with the standards found in Title 40 Code of Federal Regulations (CFR), Part 792, Good Laboratory Practices. The investigators and technicians adhered to the following guidelines: The Public Health Service Policy on Humane Care and Use of Laboratory Animals, "U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training", and the Animal Welfare Act. The studies were performed in animal facilities fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

RESULTS

GABAa receptor binding assay

TAG-MNT was tested at a single concentration of 33 μM for affinity to the GABAa convulsant site. At this concentration, TAG-MNT did not displace [^{35}S]-TBPS, that is, had no affinity for the receptor convulsant site (data not shown).

In vitro cytotoxicity of TAG-MNT

Figure 1 illustrates the levels of toxicity demonstrated by TAG-MNT in ***S. typhimurium*** and ***E. coli.*** The four bacterial strains tested demonstrated differential sensitivity to TAG-MNT. TAG-MNT was cytotoxic at 1000 μg/ml in the TA1535 and ***E. coli***. strains; cytotoxic at 500

Table 1. Results of TAG-MNT Ames assay.

Bacteria strain	-S9			+S9			Level of mutagenicity		Cytotoxicity (µg/ml)
	Control (# Revertants)	TAG-MNT (fold increase)	Positive control (fold increase)	Base line (# Revertants)	TAG-MNT (fold increase)	Positive control (fold increase)	-S9	+S9	
TA98	1.0	3.0*	41.0	1.8	3.3*	26.7*	+ 2 mg/ml	+ 2 mg/ml	500
TA100	6.4	3.5*	7.5*	3.7	0.4	10.5*	+ 1 mg/ml	negative	250
TA1535	2.4	2.7*	20*	1.6	3.4*	16	+ 2 mg/ml	+ 2 mg/ml	
TA1537	1	0.3	48*	2.9	0.3	8.1*	negative	negative	1000
E. coli	4.7	0.2	4.7*	7.4	0.7	1.8	negative	negative	2000
V. fischeri									864
Chang cells									316

* indicated significantly different from controls at $\rho \leq 0.05$.

µg/ml in the TA98 strain and at 250 µg/ml in the TA 100 strain. However, compounds can still be mutagenic even at cytotoxic concentrations (Xenometrix, 2009). The LC50 was estimated to range from 1 to 2 g/L depending on bacterial strain.

TAG-MNT appeared to be more toxic to ***V. fischeri*** and Chang liver cells in the microtox and neutral red uptake assays, respectively. The calculated EC50 15 min of TAG-MNT to ***V. fischeri*** was 864 µg/ml and the calculated IC 50 48 h to Chang liver cells was 316 µg/ml ((Table 1).

Mutagencity of TAG-MNT

The results for the mutagenicity tests are provided in Table 1. TAG-MNT was mutagenic at the limit dose of 2 g/L in three of the five strains, with or without S9 incubation. TAG-MNT was not mutagenic in the TA1537 or ***E. coli*** strains.

In all assays, the background control and the positive controls for incubations ± S9 were within the limits specified (Xenometrix 2009), that is, all assays met the criteria for validity. In several strains (Table 1), TAG-MNT was mildly mutagenic resulting in a two-fold increase only at the limit dose of 2 mg/ml, and showed a dose response only in the TA100 strain –S9.

Acute toxicity

After a single oral dose with TAG-MNT, none of the animals showed any signs of distress during the 14 day period of observation. Even at the limit dose of 2000 mg/kg, TAG-MNT showed no indication of toxicity and no sign of seizure. In the second stage of dosing, six animals received the limit dose of 2000 mg/kg; three were administered TAG-MNT in corn oil and three using the 1% methylcellulose/water vehicle. All animals in both Stages 1 and 2 survived the 14 day observation period and were then euthanized. Gross pathology observations in these animals were unremarkable.

14-Day oral repeated dose toxicity study

Clinical signs of toxicity were observed in the2000 $mg/kg^{-1}day^{-1}$ dose group. These signs included, rough coat, piloerection and lethargy at the end of the first week of dosing progressing to stained hair coat, a hunched or crouched gait, and forelimb impairment accompanied with weight loss. Five of the rats from this limit dose group lost greater than 20% of their starting body weight, became moribund, and were removed from the study and euthanized according to protocol.

The net body weight change of the animals increased similarly with time for all dose groups except for animals in the 1000 and 2000 $mg/kg^{-1}day^{-1}$ treatments. Weight gain was reduced in these two groups and was evident on the first few days of treatment (Figure 2). However, changes in weight were observed between treated and control animals only at Day 7 in the 2000 mg/kg group; five rats were severely affected (by TAG-MNT during the second week and were removed from the study. The weights of the five remaining rats recovered somewhat during the second week and the weight change was not significantly different from control (Figure 2). The net food consumption during the first week was reduced in the 1000 and 2000 mg/kg groups (data not shown).

Differences were observed in mean absolute organ weights and organ to body weight

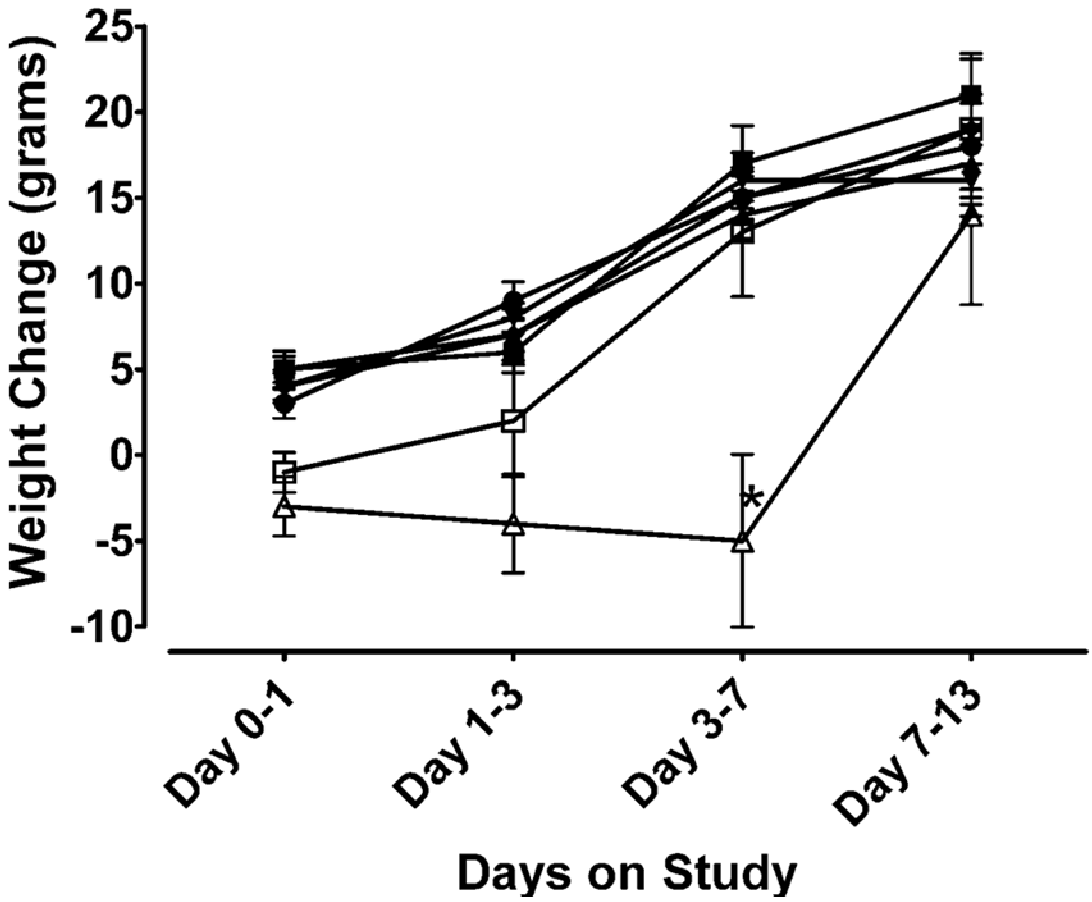

Figure 2. Body weight changes during 14-day TAG-MNT dosing study. The net body weight change of the animals increased similarly with time for all dose groups except for the two highest dose groups, 1000 and 2000 mg/kg. Weight gain was reduced in these two groups. ■ – vehicle, ▲ – 62.5 mg/kg, ▼- 125 mg/kg, ♦ - 250 mg/kg, ● – 500 mg/kg, □ – 1000 mg/kg, ○ – 2000 mg/kg. *ρ ≤ 0.05.

ratios for the kidneys, liver, adrenal glands, and spleen between the higher dose groups and the controls (Tables 2 and 3, respectively). The 500, 1000 and 2000 mg/kg^{-1}day^{-1} dose groups had elevated kidney and liver to organ/body weight ratios when compared to control animals. The adrenals and spleen had decreased organ/body weight ratios when compared to controls.

Analysis of the clinical chemistry results revealed treatment-related differences for ALB, ALT, BUN, CREA, GLU, and PHOS analytes when compared to those from samples collected from the vehicle control group (Table 4). ALT, ALB, and GLU levels were decreased relative to control values from rats in the 500, 1000, and 2000 mg/kg^{-1}day^{-1} dose groups whereas BUN, CREA, and Phos were increased (Table 4).

Treatment-related differences in the concentrations of WBC, NEU, BASO, EOS, and LYM were found when compared to the controls; however, there was no difference in the percentage of these cells in the total population (Table 5). There was no decrease in the concentration of monocytes in the WBC population and thus the percentage MONOs significantly increased. There were no treatment-related differences in RBCs, hematocrit, or other hematologic parameters (Table 5).

In the micronucleus assay, the %MN-RET in the vehicle control group was 0.28% and ranged from 0.27 to 0.34% in female rats treated with 250, 500 and 1000 mg/kg^{-1}day^{-1} of TAG-MNT in methylcellulose (Figure 3). The %MN-RET in the positive control (EMS) group was 1.16%, a marked and statistically significant ($p<0.001$) increase over that seen in the controls, indicating that the assay system was performing as expected with the known genotoxin.

Treatment with TAG-MNT did not increase %MN-RET relative to the vehicle control ($p = 0.672$). These results indicate that TAG-MNT does not induce chromosomal aberrations in rat peripheral blood and is not genotoxic in this tissue at the doses tested.

The %RET was 1.36% in the peripheral blood of the rats given the vehicle control and 0.55% in the positive (EMS) control. For rats given 250, 500 and 1000 mg/kg^{-1}day^{-1} of TAG-MNT, the %RET ranged from 1.38% at the

Table 2. Absolute weights (mg) of organs from animals treated 14 days with increasing concentrations of TAG-MNT.

Organ	Vehicle	TAG-MNT (mg/kg-day)						
	Control	0	62.5	125	250	500	1000	2000
Adrenal	Mean	0.07	0.08	0.07	0.07	0.07	0.06*	0.05*
	S.D.	0.01	0.02	0.01	0.01	0.01	0.01	0.01
Brain	Mean	1.81	1.83	1.85	1.81	1.81	1.77	1.81
	S.D.	0.10	0.08	0.07	0.06	0.05	0.10	0.09
Heart	Mean	0.80	0.83	0.82	0.81	0.81	0.69	0.76
	S.D.	0.10	0.09	0.07	0.07	0.10	0.08	0.28
Kidney	Mean	1.65	1.67	1.67	1.70	1.78*	1.82*	1.75*
	S.D.	0.13	0.14	0.12	0.13	0.13	0.19	0.16
Liver	Mean	6.66	6.60	6.68	6.77	8.03	8.75	7.89
	S.D.	0.71	0.62	0.42	0.37	0.92	0.82	0.56
Lungs	Mean	1.22	1.13	1.10	1.17	1.20	1.11	1.14
	S.D.	0.09	0.15	0.11	0.09	0.17	0.15	0.26
Ovaries	Mean	0.11	0.12	0.13	0.12	0.12	0.11	0.08
	S.D.	0.02	0.02	0.02	0.02	0.02	0.03	0.01
Spleen	Mean	0.46	0.42	0.41	0.45	0.44	0.34*	0.31*
	S.D.	0.13	0.07	0.07	0.08	0.12	0.05	0.06
Thymus	Mean	0.50	0.56	0.48	0.51	0.51	0.38	0.35
	S.D.	0.19	0.16	0.10	0.08	0.12	0.10	0.15
Uterus	Mean	0.48	0.40	0.39	0.53	0.38	0.39	0.30
	S.D.	0.21	0.07	0.05	0.19	0.07	0.10	0.10

*significantly indicated different from controls at $\rho \leq 0.05$.

Table 3. Normalized organ to body weight ratios from animals treated 14 days with increasing concentrations of TAG-MNT.

Organ	Vehicle	TAG-MNT (mg/kg-day-day)						
	Control	0	62.5	125	250	500	1000	2000
Adrenal	Mean	3.82	4.06	3.88	3.78	3.47	3.13*	3.33
	S.D.	0.45	0.69	0.62	0.54	0.33	0.32	0.68
Brain	Mean	9.60	9.95	9.92	9.65	9.70	10.17	11.14*
	S.D.	0.71	0.40	0.44	0.62	0.69	0.57	0.71
Heart	Mean	4.26	4.49	4.40	4.31	4.33	3.97	4.64
	S.D.	0.47	0.36	0.31	0.35	0.32	0.27	1.51
Kidney	Mean	8.73	9.05	8.90	9.06	9.55*	10.44*	10.79*
	S.D.	0.69	0.43	0.43	0.55	0.64	0.54	0.98
Liver	Mean	3.52	3.59	3.57	3.60	4.28*	5.00*	4.84*
	S.D.	0.25	0.28	0.18	0.13	0.24	0.29	0.28

Table 3. Contd

Lungs	Mean	6.46	6.13	5.90	6.21	6.38	6.32	6.96
	S.D.	0.41	0.61	0.49	0.42	0.53	0.44	1.37
Ovaries	Mean	5.99	6.71	7.01	6.43	6.39	6.20	5.08
	S.D.	1.20	1.17	1.03	0.92	1.07	1.58	1.08
Spleen	Mean	2.43	2.29	2.20	2.37	2.35	1.94*	1.89
	S.D.	0.56	0.30	0.30	0.38	0.48	0.17	0.39
Thymus	Mean	2.65	3.04	2.56	2.73	2.74	2.17	2.14
	S.D.	0.95	0.75	0.57	0.36	0.63	0.70	0.83
Uterus	Mean	2.60	2.16	2.09	2.81	2.03	2.18	1.81
	S.D.	1.25	0.37	0.26	1.06	0.42	0.48	0.64

* indicated significantly different from controls at $\rho \leq 0.05$.

Table 4. Clinical plasma chemistry results for female rats exposure orally to TAG-MNT for 14-days.

Organ	Vehicle	TAG-MNT (mg/kg-day)						
	Control	0	62.5	125	250	500	1000	2000
ALB	Mean	3.1	3.2	3.2	3.2	3.2	3.1	2.6
(g/dl)	S.D.	0.2	0.2	0.1	0.2	0.3	0.2	0.3
ALKP	Mean	209.6	216.9	215.6	213.2	223.2	168.0	204.4
(U/L)	S.D.	42.7	47.4	35.5	26.8	41.5	52.4	60.6
ALT	Mean	50.2	48.8	43.7	39.1*	43.0	31.4*	22.6*
(U/L)	S.D.	5.4	6.1	4.2	5.3	6.1	8.5	5.0
BUN	Mean	17.3	18.1	18.8	19.0	21.0	24.4*	25.0*
(mg/dl)	S.D.	2.9	1.7	3.4	3.3	4.6	1.8	4.8
Ca	Mean	10.4	10.1	10.4	10.4	10.4	10.3	10.1
(mg/dl)	S.D.	0.2	0.4	0.3	0.4	0.3	0.3	0.4
CHOL	Mean	44.0	49.3	42.4	48.3	48.2	37.3	33.6
(mg/dl)	S.D.	16.0	12.5	14.3	8.5	21.8	15.5	5.8
CREA	Mean	0.5	0.5	0.5	0.6	0.6	0.6*	0.8*
(mg/dl)	S.D.	0.1	0.1	0.1	0.1	0.1	0.1	0.1
GLOB	Mean	2.6	2.7	2.7	2.7	2.7	2.5	2.5
(g/dl)	S.D.	0.1	0.2	0.1	0.2	0.1	0.2	0.2
GLU	Mean	146.3	136.2	134.0	133.5	119.2*	119.7*	118.0*
(mg/dl)	S.D.	13.9	17.6	17.8	13.6	20.9	10.9	5.0
LDH	Mean	2038.9	3209.9	2423.2	1667.0	2717.1	2889.2	3565.8
(U/L)	S.D.	1038.7	1647.1	1493.2	792.5	1722.1	1508.6	2306.3

Table 4. Contd

PHOS	Mean	7.7	7.5	7.6	8.1	8.4	9.5*	10.2*
(mg/dl)	S.D.	0.4	0.5	0.4	0.9	1.0	1.4	1.7
TBIL	Mean	0.1	0.3	0.2	0.1	0.2	0.3	0.3
(mg/dl)	S.D.	0.0	0.3	0.1	0.0	0.1	0.2	0.5
TP	Mean	5.7	5.9	5.9	5.9	5.9	5.6	5.1
(g/dl)	S.D.	0.2	0.3	0.2	0.3	0.3	0.3	0.4
Na	Mean	139.3	138.9	139.4	140.4	138.7	137.1	134.4
(mmol/l)	S.D.	1.3	1.8	2.3	2.4	2.2	3.0	1.1
K	Mean	4.5	4.8	4.6	4.6	4.4	4.6	5.5
(mmol/L)	S.D.	0.5	0.4	0.5	0.8	0.6	0.8	1.0
Cl	Mean	105.4	105.4	104.9	104.6	103.2	101.3	101.2
(mmol/L)	S.D.	2.2	1.0	2.0	2.0	2.5	1.6	2.7

* indicated significantly different from controls at $\rho \leq 0.05$.

Table 5. Changes in red blood cell count, hematocrit, hemoglobin, white blood cell count, and proportion of white blood cell types as a function of oral TAG-MNT exposure in female rats.

	Vehicle	**TAG-MNT (mg/kg-day)**						
	Control	**0**	**62.5**	**125**	**250**	**500**	**1000**	**2000**
WBC	Mean	16.1	11.2*	9.3*	12.6	10.2*	7.3*	6.0*
(K/❍L)	S.D.	3.2	4.4	1.8	3.2	4.3	3.2	5.7
NEU	Mean	1.3	0.8	0.8	0.7	0.8	0.5*	0.2*
(K/❍L)	S.D.	1.0	0.4	0.4	0.2	0.4	0.3	0.2
(%N)	Mean	8.3	8.2	8.7	5.6	8.1	7.6	5.4
	S.D.	6.4	6.1	3.3	2.1	2.1	4.1	1.9
LYM	Mean	13.9	9.8	7.2*	11.2	8.7*	6.2*	5.2*
(K/❍L)	S.D.	3.0	4.2	2.7	3.0	3.6	2.8	5.3
(%L)	Mean	85.9	86.0	84.8	88.6	85.3	85.1	82.3
	S.D.	7.4	6.5	4.3	2.6	3.5	6.0	8.8
MONO	Mean	0.5	0.3	0.3	0.4	0.4	0.3	0.4
(K/❍L)	S.D.	0.1	0.1	0.1	0.2	0.2	0.2	0.3
(%M)	Mean	2.9	2.7	3.2	3.1	3.3	4.4*	8.2*
	S.D.	0.7	0.7	0.7	0.7	1.4	1.8	3.5
EOS	Mean	0.3	0.1	0.1	0.1	0.1	0.05*	0.04
(K/❍L)	S.D.	0.5	0.0	0.1	0.0	0.0	0.0	0.0
(%E)	Mean	1.1	1.0	1.2	0.9	1.1	0.8	0.9
	S.D.	0.3	0.3	0.4	0.4	0.4	0.4	0.5

Table 5. Contd

BASO (K/❍L)	Mean	0.3	0.2	0.2	0.2	0.2	0.16*	0.1
	S.D.	0.1	0.2	0.1	0.1	0.1	0.1	0.0
(%B)	Mean	1.9	2.1	2.1	1.8	2.2	2.1	3.2
	S.D.	0.5	0.6	0.6	0.4	0.7	0.8	2.9
RBC (M/❍L)	Mean	7.2	7.1	7.2	7.1	7.0	7.2	7.7
	S.D.	0.3	0.2	0.2	0.5	0.5	0.3	0.2
HGB (g/dL)	Mean	14.1	13.6	14.0	13.9	13.7	13.5	14.0
	S.D.	0.5	0.4	0.5	1.0	0.5	0.6	0.1
HCT (%)	Mean	41.5	40.0	41.1	41.0	40.0	40.1	41.4
	S.D.	1.6	1.2	1.4	2.8	2.6	1.6	1.0
MCV (fL)	Mean	57.7	56.4	56.9	57.5	57.4	55.7	54.1
	S.D.	1.1	1.7	1.9	1.6	2.4	1.8	3.0
MCH (pg)	Mean	19.6	19.2	19.3	19.4	19.8	18.7	18.3
	S.D.	0.3	0.8	0.6	0.6	1.4	0.7	0.4
MCHC (g/dL)	Mean	33.9	34.0	33.9	33.8	34.4	33.6	33.9
	S.D.	0.3	0.5	0.2	0.2	1.8	0.5	1.1
RDW (%)	Mean	14.4	15.1	14.2	14.9	14.2	15.3	17.4
	S.D.	0.6	0.7	0.5	0.7	0.8	0.7	0.0
PLT (K/❍L)	Mean	1073.6	1071.1	1062.6	1035.8	1084.0	848.1	1086.5
	S.D.	208.6	107.4	180.0	316.7	216.8	426.6	88.4
MPV (fL)	Mean	5.4	5.8	5.4	5.2	5.2	5.4	5.3
	S.D.	0.5	0.4	0.2	0.4	0.2	0.4	0.1

*Significantly different from Vehicle-treated animals.

low dose to 1.09% at the high dose (Figure 4). There were no differences in the frequency of reticulocytes in the peripheral blood among treated and vehicle control groups ($p = 0.401$). These results suggest that erythropoiesis in the bone marrow was not affected by treatment with TAG-MNT.

Histopathology was performed on liver and kidney tissues in all dose groups ($n = 5$) with preference to animals showing signs of organ weight changes or organ discoloration. No adverse events were observed in kidney. However, dose-related adverse events were observed in the liver tissues (Figure 5). Observed lesions included: Single-cell necrosis, macrocytic and microcytic cytoplasmic vacuolization of hepatocytes, inflammation, hepatocyte degeneration, centrilobular hepatocyte hypertrophy, increased mitoses, megakaryocytic hepatocytes, and bile duct hyperplasia. There was a dose-relationship of single-cell necrosis with increasing incidence and severity at the three highest doses, 500, 1000 and 2000 $mg/kg^{-1}day^{-1}$ (Table 6). Lymphocytic and acute inflammation was noted in all treated groups, most evidently in the three highest dose groups, but not in the control group.

Hepatocyte degeneration was observed in one animal in each of the two highest-dose groups. Observations of centrilobular hypertrophy was also dose-related, with the incidence and severity consistent with increasing dose (periportal hypertrophy was present only at the highest dose). Megakaryocytic hepatocytes and increased heaptocyte mitoses were noted in several animals, indicative of a response to injury. Granulomatous inflammation or infiltration present in the control and treated groups was an incidental finding not considered to be related to treatment.

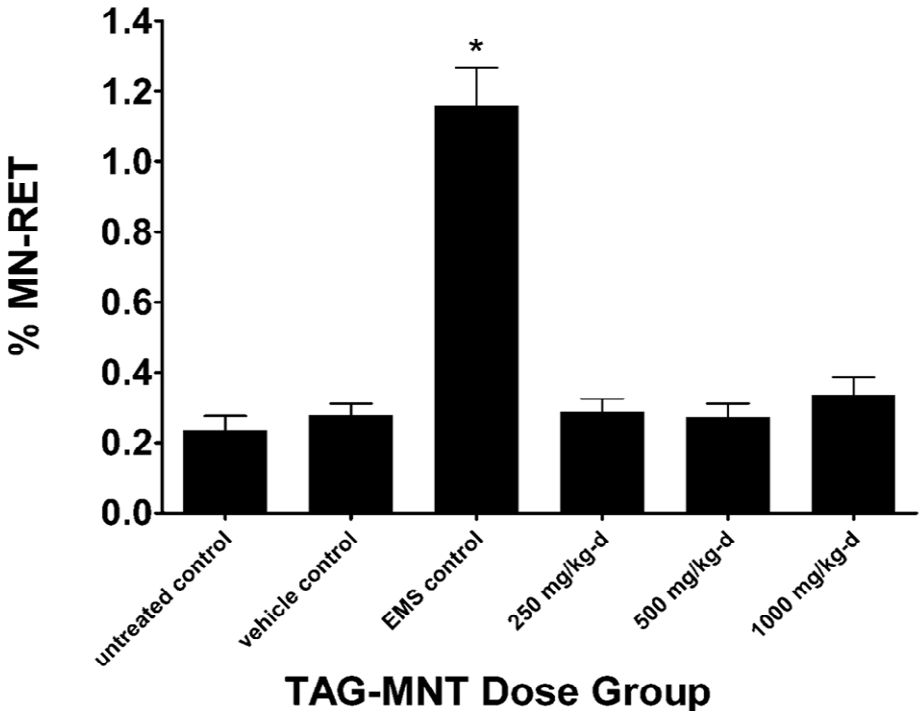

Figure 3. Percent micronucleated reticulocytes in female rats orally dosed with TAG-MNT for 14-days. There were no differences in the frequency of micronucleated reticulocytes between the vehicle control group and TAG-MNT dose groups. Treatment with the positive control (EMS) increased the frequency of micronucleated reticulocytes.

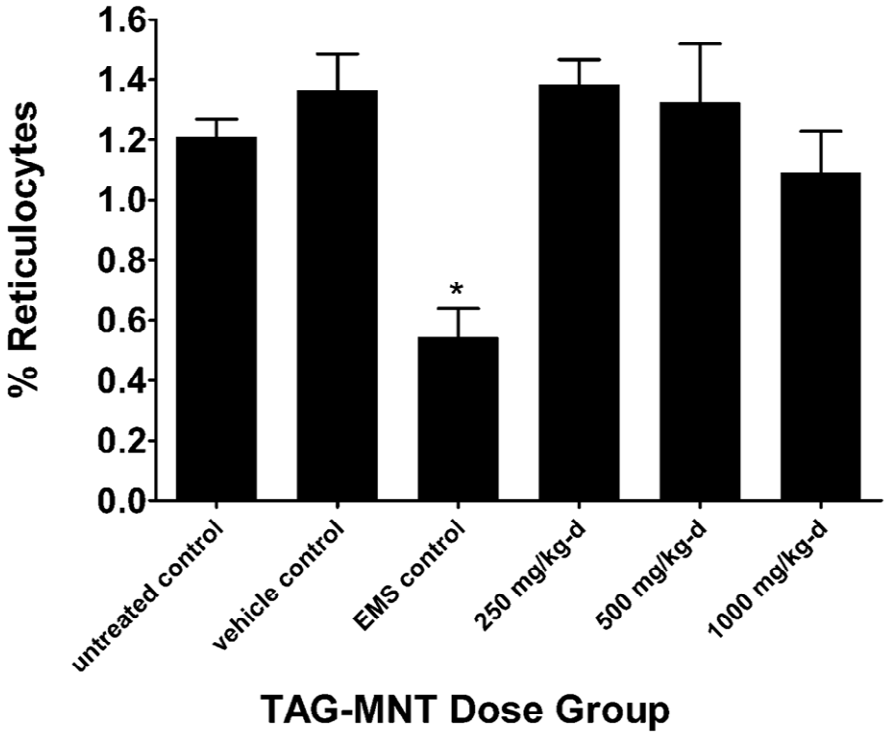

Figure 4. Percent reticulocytes in female rats orally dosed with TAG-MNT for 14-days. The frequency of reticulocytes did not differ between the vehicle control group and TAG-MNT dose groups. Treatment with the positive control (EMS) reduced the frequency of reticulocytes.

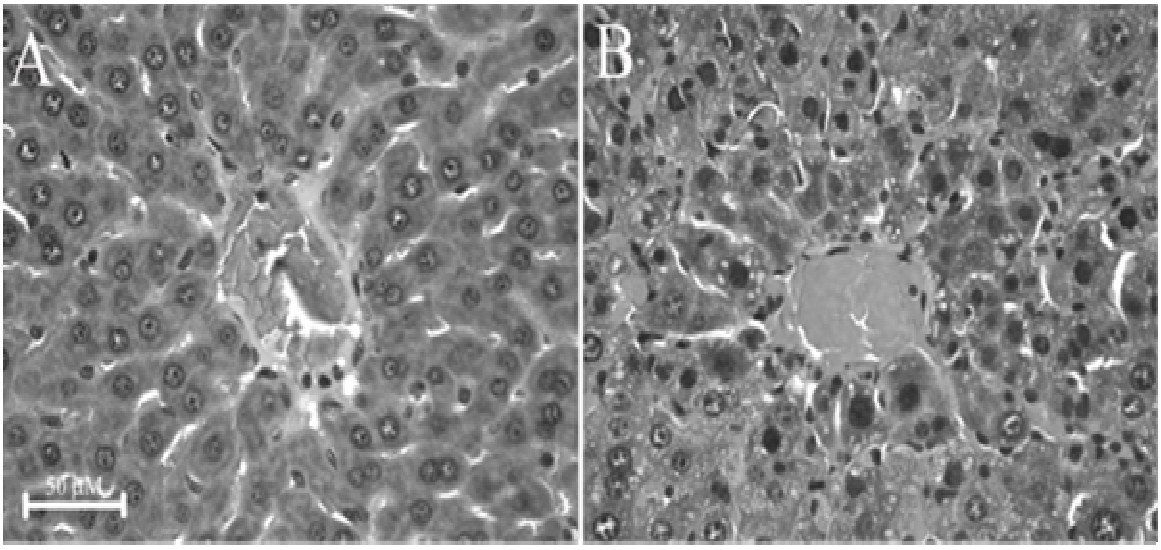

Figure 5. Histopathology of Liver. Micrograph of a central vein from a vehicle-treated animal (control, A) and from a high dose rat receiving 2000 mg/kg-d (B) for 14 days. Hepatocyte degeneration and necrosis is shown along with megakaryocytoses, cytoplasmic vacuolization, inflammation, and centrilobular hepatocyte hypertrophy. Scale bar equals 50 µm.

Table 6. Histological hepatic observations of oral TAG-MNT exposure in female rats.

Observation	TAG-MNT (mg/kg)						
	0	62.5	125	250	500	1000	2000
Necrosis, single cell	1/5	2/5	0/5	2/6	3/5	2/5	5/5
Trace	1/5	1/5	0/5	2/6	1/5	0/5	0/5
Mild	0/5	1/5	0/5	0/6	2/5	2/5	3/5
Moderate	0/5	0/5	0/5	0/6	0/5	1/5	2/5
Centrobular hypertrophy	0/5	0/5	0/5	0/6	1/5	2/5	3/5
Mild	0/5	0/5	0/5	0/6	1/5	2/5	2/5
Moderate	0/5	0/5	0/5	0/5	0/5	0/5	1/5
Vaculolar change	3/5	4/5	2/5	4/6	6/5	5/5	4/5
Trace	3/5	2/5	2/5	0/6	0/5	4/5	1/5
Mild	0/5	2/5	0/5	4/6	4/5	2/5	2/5
Moderate	0/5	0/5	0/5	0/5	2/5	1/5	2/5
Severe	0/5	0/5	0/5	0/5	0/5	0/5	1/5

DISCUSSION

Testing new compounds requires an integrative, holistic approach that is phased proportional with the level of investment or value of the new substance and how it will be used (American Society for Testing and Materials (ASTM), 2008). Many assays are available that provide value in assessing the risk from substance exposure. However, all assays have drawbacks, shortcomings, and uncertainties when applying assay results in a risk assessment paradigm. Therefore, the results of many tests can help provide a more complete evaluation of relative impact and serve to reduce levels of certainty while maintaining costs consistent with the level of efforts devoted to the system or platform. Here we compare there sults of these tests on TAG-MNT relative to a compound with a similar function, that is, RDX.

Cytotoxicity of TAG-MNT was observed in ***S. typhimurium*** and ***E. coli*** after a 90 min exposure of concentrations greater than 250 mg/L with an estimated

LD50 of 1 g/L. Data from ***V. fischeri*** exposures to TAG-MNT suggest an increased sensitivity than the other bacteria species tested (EC50 15 min = 864 µg/ml). However, using US Fish and Wildlife Service aquatic toxicity criteria, TAG-MNT is considered "Practically Nontoxic" as the EC50 is greater than 100 µg/ml (US Fish and Wildlife Service (USF&WS) 1984). ***V. fischeri*** are frequently more sensitive to xenobiotics than other aquatic organisms and thus, the results from Microtox® tests are often useful screens in the assessment of relative toxicity to aquatic organisms (Dutka and Kwan, 1981; Argese et al., 1998; Codina et al., 1993; McFeters et al., 1983; Choi et al., 2004). Thus, TAG-MNT is predicted to have a low probability for inducing aquatic toxicity.

Change in uptake of neutral red in Chang liver cells was also observed as a result of TAG-MNT exposure. Sufficient data have been obtained from the NRU studies that it can be used as a predictor of ***in vivo*** toxicity (Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) et al., 2006). The results of TAG-MNT in the NRU assay (IC 50 48 h = 316 µg/ml) predicted that TAG-MNT would have an oral LD50 of 900 mg/kg in rat. However, the present ***in vivo*** study found that acute oral exposures of up to 2000 mg TAG-MNT/kg failed to produce death or other overt toxicity in Sprague-Dawley female rats.

TAG-MNT showed a weak positive mutagenic response both with and without S9 metabolism at the highest, limit dose. Although meeting the criteria for a positive response for mutagenicity, the number of mutant reversions was less than 3.5-fold above background; the positive controls for the ***S. typhimurium*** strains were greater than 10-fold above background. However, OECD guidelines indicate that mutagenicity testing is valid only above cytotoxic concentrations (OECD, 1997). Thus, TAG-MNT is not considered mutagenic as signs of mutagenicity only occur at concentrations 2 to 4 times above the cytotoxic concentration (Table 1).

Unlike other tetrazole derivatives (Luttjohann et al., 2009; Sharma et al., 1994; Squires et al., 1984), TAG-MNT did not have affinity for the GABAa receptor in the ***in vitro*** binding assay. This result predicted that TAG-MNT would probably not induce convulsions like RDX when administered to rats. In fact, oral administration of TAG-MNT did not cause seizure or any other sign of acute toxicity up to the limit dose of 2000 mg/kg. However, daily administration for 14-days resulted in effects to other targets. TAG-MNT primarily affected the liver causing hepatocyte hypertrophy, inflammation, and increased liver weight associated with weight loss and animal morbidity. The histopathologic observations combined with other evidence suggests a No observed adverse effect level (NOAEL) and a lowest observed adverse effect level (LOAEL) of 250 and 500 $mg/kg^{-1}day^{-1}$, respectively.

The statistically significant increases in BUN and CREA in the high dose groups are consistent with dysfunction of the kidneys. However, this was not supported by evidence of histopathology in the kidney tissues examined. The significant decrease in ALT is counterintuitive as a marker of liver dysfunction as increases in free (systemic) ALT concentrations are usually attributed to increased liver parenchymal cell necrosis. Potential causes of decreased serum activities of ALT are reported to include: Decreased hepatocellular production or release of the enzymes; inhibition or reduction of the enzyme activity; or interference with the enzyme assay (PSD Guidance Document, 2007). Decreases in ALT have previously been reported in other chemical-induced, liver pathology models (Waner and Nyska, 1991). However, the most widely recognized cause is considered to be a negative effect, directly or indirectly, on pyridoxal 5'-phosphate (ECETOC 2002; PSD Guidance Document, 2007).

There was a reduction in white blood cell concentrations in rats receiving TAG-MNT oral doses of 500 $mg/kg^{-1}day^{-1}$ and above. This included a genera l decrease in number of all subpopulations of WBCs, particularly neutrophils and lymphocytes, and thus no change in the overall percentage of these cells. Typically, these reductions are a result of increased levels of circulating stress proteins, e.g. glucocorticoids, an observation common in stressed animals (Burns-Nass et al., 2001).

The current USEPA regulatory values for RDX are based on prostatic inflammation in rodents as a result of chronic oral exposures (Levine et al., 1983), and hepatocellular carcinomas in only female mice (Lish et al., 1984; McLellan et al., 1992; Parker et al., 2006). However, the primary toxicity of RDX is its induction of epileptiform seizure following oral ingestion (Burdette et al., 1988; Kasuske et al., 2009; Stone et al., 1969). The mechanism of seizure induction has recently been shown to involve blockage of chloride flux through the GABAa receptor ligand-gated channel as a result of RDX binding to the convulsant site of the receptor (Williams et al., 2010). TAG-MNT did not have affinity for the GABAa receptor when tested in an ***in vitro*** binding assay, and oral administration of TAG-MNT to female rats did not cause seizure or any other sign of acute toxicity up to the limit dose of 2000 mg/kg. In contrast, the LD50 of RDX in rat is 60 mg/kg where death always follows induction of seizure (USACHPPM. 2006). Thus, the acute oral toxicity of TAG-MNT is orders of magnitude less than RDX.

As a group, relatively few toxicity data are available for tetrazoles. Tetrazoles have been associated with convulsant activity similar to that found with RDX where the GABA-chloride ionophore receptor complex is inhibited (Sharma et al., 1994; Squires et al., 1984; Williams et al., 2010; Luttjohann et al., 2009). The present study found daily administration of TAG-MNT for 14-days resulted in adverse effects primarily in the liver resulting in hepatocyte hypertrophy, inflammation, increased liver weight and morbidity; no evidence of neurotoxcity was observed nor was there activity associated with the GABA

picrotoxin convulsant site. These data suggest that TAG-MNT primarily affects blood conditioning organs; though additional study is needed to further understand the complete spectrum of adverse effects from those exposed through manufacture and use.

ACKNOWLEDGMENTS

This work was supported by the US Army Environmental Quality Technology Program through the U.S. Army Research, Development and Engineering Command, Environmental Acquisition and Logistics Sustainment Program.

The authors would like to acknowledge the support and encouragement of Erik Hangeland of the U.S. Army Research, Development and Engineering Command, Environmental Acquisition and Logistics Sustainment Program. We also thank Pat Beall, Anne MacLarty, Will McCain, Valerie Adams, Terry Hanna, John Houpt, and Mark Way for providing technical support. Particular acknowledgment goes to Martha Thompson for collecting LABCAT® data and generating the appropriate reports. We also thank Desmond Bannon and Cindy Landgren for their critical review of the manuscript.

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